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OFFICE OF NAVAL RESEARCH

Contract #N00014-78-C-0713

Task No. NR 133-994

FINAL REPORT

Development of a Methodology for
the Rapid Detection of Coliform
Bacteria

by

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27 February 1981

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE		READ INSTRUCTIONS BEFORE COMPLETING FORM
1. REPORT NUMBER	2. GOVT ACCESSION NO. $AD-A096$	3. RECIPIENT'S CATALOG NUMBER 008
4. TITLE (and Subtitle) Development of a Methodology for the Rapid Detection of Coliform Bacteria		5. TYPE OF REPORT & PERIOD COVERED Final Report Sept.1, 1979–Dec.31,1980
		6. PERFORMING ORG. REPORT NUMBER
7. AUTHOR(s) F. Pinteno, E. Findl		8. CONTRACT OR GRANT NUMBER(s) Contract N00014-78-C-0713
9. PERFORMING ORGANIZATION NAME AND ADDRESS BioResearch, Inc. 315 Smith Street Farmingdale, NY 11735		10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS
11. CONTROLLING OFFICE NAME AND ADDRESS Office of Naval Research 800 N. Quincy Street Arlington, VA 22217		12. REPORT DATE February 27, 1981
		13. NUMBER OF PAGES 40
14. MONITORING AGENCY NAME & ADDRESS(if different from Controlling Office)		15. SECURITY CLASS. (of this report) Unclassified
		15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)

Unlimited Distribution

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

β-D-galactosidase, coliform bacteria, rapid detection, w/o emulsions, fluorescence flow cytometer, encapsulation

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Work has continued on the development of a rapid method for the enumeration of coliform bacteria, based on the hydrolysis of a fluorogenic substrate by the enzyme β-D-galactosidase. A validation of the basic manual technique was completed using both laboratory and field samples. A correlation between the percentage of microdroplets and the log of the bacterial concentration was established in both cases.

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A semiautomated version of the manual technique was rendered feasible through the use of two fluorescent dyes (fluorescein and ethidium bromide) in the detection of microdroplets containing bacteria, as well as those that did not. The use of this system, with a Zeiss microscope equipped with a Farrand spectrum analyzer and motorized scanning stage, provided a means by which slides could be analyzed without direct visual observation. As in the case of the manual technique, however, detection was limited to concentrations $\geq 10^5$/ml.

The ICP-22 fluorescent flow cytometer (Ortho) arrived relatively late (end of February) due to manufacturing delays in Germany. Extensive studies conducted with E. coli Neotype induced for β-D-galactosidase activity revealed that fluorochromasia, per se, was beyond the resolution capabilities of the instrument. Signals that were initially observed were later determined to be the result of interference signals arising from microairbubbles. Experimental evidence indicated that fluorescein efflux from bacterial cells was too rapid to allow any significant accumulation and thereby minimized the fluorochromasia effect.

The use of w/o (water in oil) emulsions was successfully employed in the entrapment of bacteria and the containment of fluorescein. A method was developed by which relatively stable emulsions having microdroplet diameters $< 10\mu$ could be formed under simple vortexing conditions. Although initial instrument incompatibility problems occurred, a redesigning of the instrument's fluid delivery system led to their eventual elimination.

At this point in time, the detection rate of E. coli Neotype emulsions having a concentration $\sqrt{10^7}$/ml falls within the range of 0.2-0.3%. At concentrations $< 10^3$/ml there is no detection. These findings indicate that the detection of sample signals arising from relatively high concentrations of E. coli Neotype are the result of multiple numbers of bacteria entrapped within microdroplets. At lower concentrations, individual bacteria are entrapped necessitating considerably longer times (>4 hrs) to elaborate comparable levels of fluorescence.

In short, the feasibility of the microemulsion technique in the rapid detection of coliform bacteria is currently hampered by a relatively low S/N ratio separation ($\sim$2:1). A ratio of at least 5:1 will have to be attained to accurately quantify bacterial concentrations with any degree of reproducibility.

FOREWARD

This report has been prepared for the Office of Naval Research (ONR) and the U.S. Army Mobility and Equipment Research and Development Command (MERADCOM) in accordance with the requirements of Contract N00014-78-C-0713 as revised September 1979. The period of performance of the program was September 1, 1978 to March 30, 1979 and September 1, 1979 to December 31, 1980. Cutbacks in pollution funding by the Naval Material Command resulted in both program delays and reduction in staffing levels recommended for the project. This report describes the progress made during the period March 1 to December 31, 1980.

The objective of the program was to extend the rapid coliform detection procedures from 10^5 coliforms/ml down to 2 coliforms per ml utilizing a fluorescence flow cytometric counting technique. The program was conducted by the Bioelectrochemistry Division of BioResearch under the direction of Eugene Findl. Technical management of the program was performed by Frank Pinteno.

Technical monitors for the program were Dr. F. Santana (LCDR) of ONR and M. Pressman of MERADCOM. Their assistance and guidance is gratefully acknowledged.

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INTRODUCTION

A major goal of the feasibility study to "Develop a Methodology for the Rapid Detection of Coliform Bacteria", contract N-00600-77-C-1163, has been to demonstrate that fecal and/or non-fecal coliform bacteria can be detected and quantified in approximately one hour. During the first phase of the study, a manual fluorescent dye technique was developed that (a) demonstrated that coliforms could be detected in $\simeq$ 1 hour in a milieu containing other type of microorganisms (b) demonstrated that fecal coliforms could be distinguished from non-fecal coliforms and (c) demonstrated that the manual technique would be useful for quantitation only at coliform densities > 10^5/ml. Phase 2 of the program, initiated after a program delay of $\simeq$ 6 months, was initiated to determine the feasibility of a fluorescence flow cytometric technique to lower the quantitation level to a bacterial concentration of 2/ml.

After a 5 month additional delay awaiting delivery of a flow cytometer, experimental effort was re-initiated to optimize the optical sensitivity of the cytometer and to demonstrate the feasibility of automatically detecting and quantitating coliforms. Preliminary experiments centered on attempts to utilize bacterial membranes rather than water in oil microdroplets to contain a fluorescent dye released by coliforms during hydrolysis of an enzyme-specific substrate. Initial results looked promising. It was later shown, however, that what were initially thought of as coliform counts were in reality micro air bubbles. These air bubbles were released in the sample stream by the action of the cytometer vacuum pumping system used to bring the sample into the cytometer. In essence, air dissolved in the sample flow system was liberated due to a lowering of the absolute pressure in the flow tubing.

Once the cause of the problem was realized, various mechanical modifications were made to the flow cytometer to substitute a pressurized flow system for the vacuum system. Initially air pressure was used as the driving force in a revised flow system. With the air pressurized system, air bubbles were eliminated. However, shortly thereafter, it was realized that our efforts at utilizing bacterial membranes to contain the fluorescent hydrolysis product, i.e., fluorescein, were ineffective. The decision was then made to return to the initial concept of water in oil microdroplets to contain both the bacteria and the fluorescence produced by hydrolysis.

In the manual mode of operation, microdroplets are produced by spraying an aqueous aerosol onto a microscope slide containing silicone oil of approximately the same density as water. Simple sample aeration onto oil is unsuitable for use in a fluorescence flow cytometer. Therefore, a new approach to microbubble formation was needed. An obvious approach is to emulsify the aqueous sample containing bacteria using a water in oil emulsion.

Attempts at simple homogenization of water and silicone oil in a ratio of 1:9 using a high speed, high shear mixer indicated that we could indeed produce microdroplets in the desirable size range, i.e. $\simeq 20\mu$ diameter. However, microdroplet agglomeration occurred in a short time period, generally < 10 minutes. Our effort was then shifted to evaluating surfactants to stabilize the emulsion so that we could achieve stability for at least 30 minutes. This would seem to be a trivial problem. We soon found that there were few surfactants available for use with a water in silicone oil mixture. Discussions with several silicone oil manufacturers gave us certain clues as to potential surfactant candidates.

After a number of trial and error experiments, we came up with a combination of surfactants that were apparently satisfactory. Trial runs were then made using microdroplets of an aqueous control solution of fluorescein. At first, all went well, the microdroplets could be readily seen for the first time through the eyepiece of the cytometer. However, a new pulsing phenomenon, similar to the air bubble problem was noted. The cause was determined to be both an interfacial phenomenon and a flow problem. Briefly, water is used in 2 flow systems of the cytometer. These are called the sheath and transverse flow systems. Where the silicone oil in the sample-flow merged with the sheath stream, discrete globules of sample were formed. These passed the fluorescence detector at regular intervals giving rise to pulsed electrical signals.

A variety of flow parameters were varied to eliminate the problem. The eventual solution proved to be the elimination of the gas pressurization flow system and its supplantation by a positive pressure multi-channel peristaltic pump. In addition, the flow characteristics of the silicone oil were modified by using one of lower viscosity. We also determined that simple vortexing of the new silicone oil-water mixture produced uniform microdroplets $\simeq 10\mu$ in diameter.

Overall, the result of the instrumental modifications was the establishment of a stable flow system with no pulsing. Subsequent analyses conducted with relatively high concentrations of E. coli Neotype ($\sim 10^7$/ml) demonstrated detection of only a proportion of the theoretical concentration. This was considered to be the result of multiple numbers of E. coli entrapped within microdroplets being detected as one. The use of lower concentrations of E. coli Neotype (~ 100/ml and 1000/ml) demonstrated a lack of correlation with the theoretical. In these cases the observed detection was too high. As a result of these findings, negative control emulsions of the substrate were prepared and run through the instrument. The findings demonstrated that background signals (false positives) were being elaborated by trace amounts of fluorescein arising from autolysis. This problem was not previously encountered due to a dilution step (1:100) incorporated in the original procedure to minimize the interference of free fluorescein. The present

emulsion technique does not readily afford an opportunity at which a dilution of the sample can be performed. Another contributing factor is the relatively more intense emission provided by a 100W mercury-arc excitation source over the 100W halogen lamp used in prior efforts with a fluorescence microscope.

Measures taken to further purify stock crystals of the substrate using such approaches as deionized H_2O extraction, recrystallization, and 20mM KH_2PO_4 buffer extraction, ultimately yielded a considerably purer substrate. Negative control emulsions of the new preparation demonstrated virtually no background signals (false positives). A parallel study was conducted using two concentrations of optimally induced E. coli Neotype ($\sim10^3$/ml and 10^7/ml). Analysis of the samples indicated that the instrument was not responding to the levels of fluorescein produced by the coliforms. Additional tests with varying levels of fluorescein encapsulated in microemulsions indicate that the sensitivity of the instrument is not optimized. Fluorescence of microbubbles can be seen visually in the instrument's eyepiece and yet is not being registered as a count. Further evaluations of the technique should concentrate on improving instrument sensitivity.

TECHNICAL DISCUSSION

Details of the revised instrumentation developed, fluorescence analysis procedure modifications, and new techniques investigated during the contractual period are discussed herein.

Continued Validation of Manual Technique

The procedure adopted for the rapid detection of coliform bacteria is based on the research work of Dr. B. Rotman, of Brown University, who developed techniques for the detection of single molecules of the enzyme β-D-galactosidase. The biochemical reactions exploited in the rapid detection method are 1) induction of β-D-galactosidase within the E. coli by the inducer isopropyl thio β-D-galactopyranoside (IPTG), 2) transport of the substrate fluorescein-di-β-galactopyranoside (FDG) into bacterial cells, and 3) hydrolysis of the FDG to liberate the fluorescent dye fluorescein. Although fluorescein is highly fluorescent and can be detected at low concentrations, the amounts of fluorescein that can be produced by a single induced bacterium will be rapidly diluted by the water surrounding that bacterium. Rotman solved the dilution problem by containing one, or a small number of E. coli within microdroplets of water produced by an atomizer sprayed onto silicone coated slides. Diffusion of FDG into bacterial cells and the diffusion of liberated fluorescein out can be accelerated by treating the cell suspension with isoamyl alcohol to perforate the cell membrane. Figure 1 illustrates the manual technique that was originally investigated.

The manual rapid coliform detection results were routinely compared to the coliform numbers determined by plate counts on nutrient agar. To calibrate the technique against standard methods for coliform numbers, the number of E. coli Neotype in five samples ranging in cell density from 10^5 to 10^9 organisms per ml were counted using both the Most Probable Number (MPN) multiple tube fermentation technique and plate counts.

A plot of the percentage of fluorescent droplets per field of view against the cell density of the E. coli Neotype determined by plate counts is shown on Figure 2. Each determination took 1 to 1-1/2 hours to complete. This time was divided into the dilution of E. coli suspension (2 minutes), IPTG induction (30 minutes), centrifugation and resuspension (10 minutes), addition of FDG and isoamyl alcohol (2 minutes), spraying (1 minute), incubation of the slide (15 minutes) and counting of 10 fields of view on each slide (20 minutes).

To ensure that the rapid coliform detection method could be considered comparable to the MPN multiple-tube fermentation technique for the counting of total coliform numbers, the counts achieved by plate counts of E. coli Neotype on nutrient agar were compared to the counts in multiple-fermentation tubes (Figure 3). A linear relationship between the two counting techniques for E. coli Neotype was demonstrated.

N₂

Atomizer

Capillary Tube Filled With Bacterial Suspension

Diameter at Tip ≅ 20 μ

Diameter of Well ≅ 18 mm

Parafilm

Silicaid Coated Slide

Well Holding 2-3 Drops Silicone Oil

View Under Microscope

Fluorescent Droplet ≅ 15-20 μ

Bacteria Contained Within Droplet
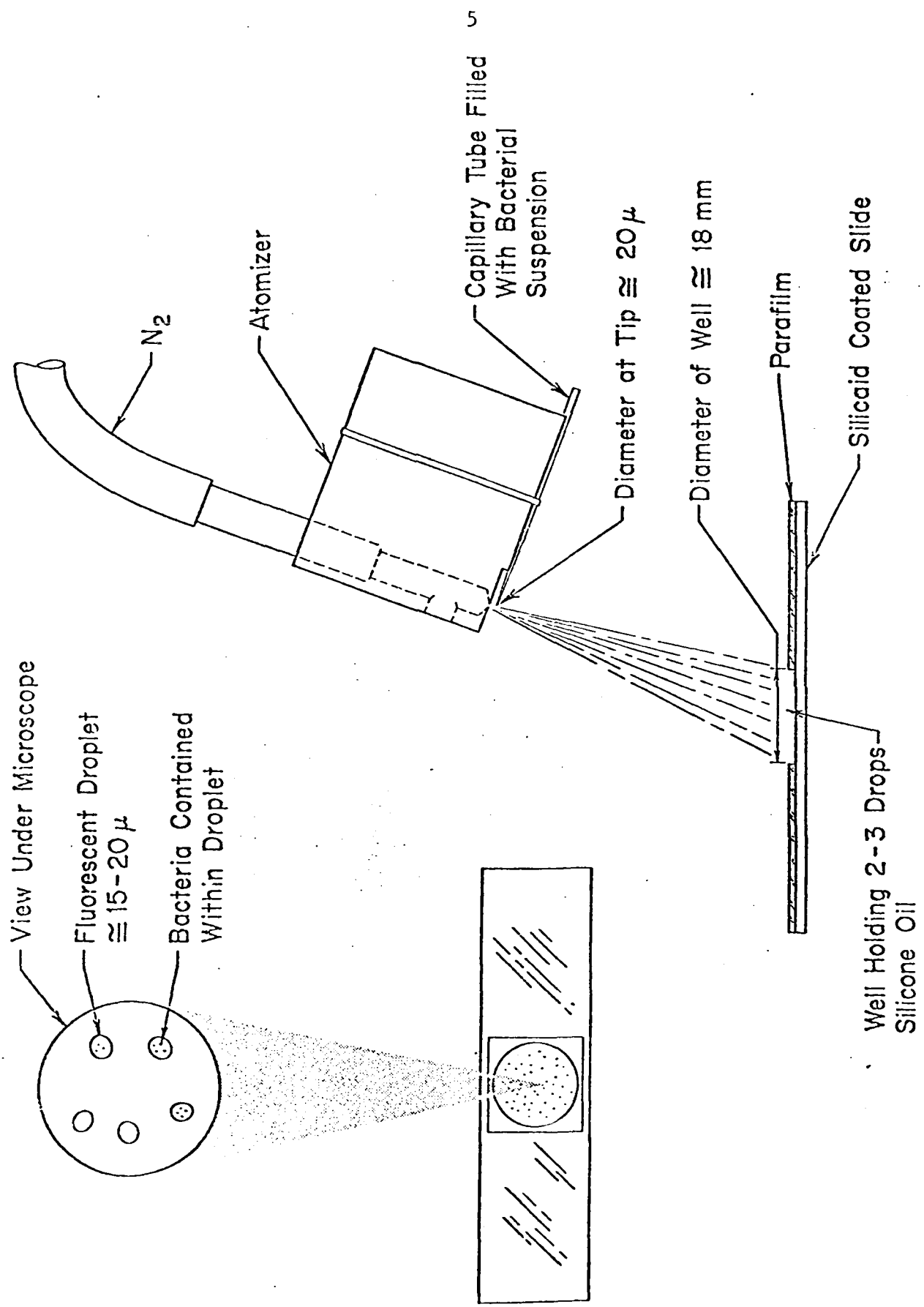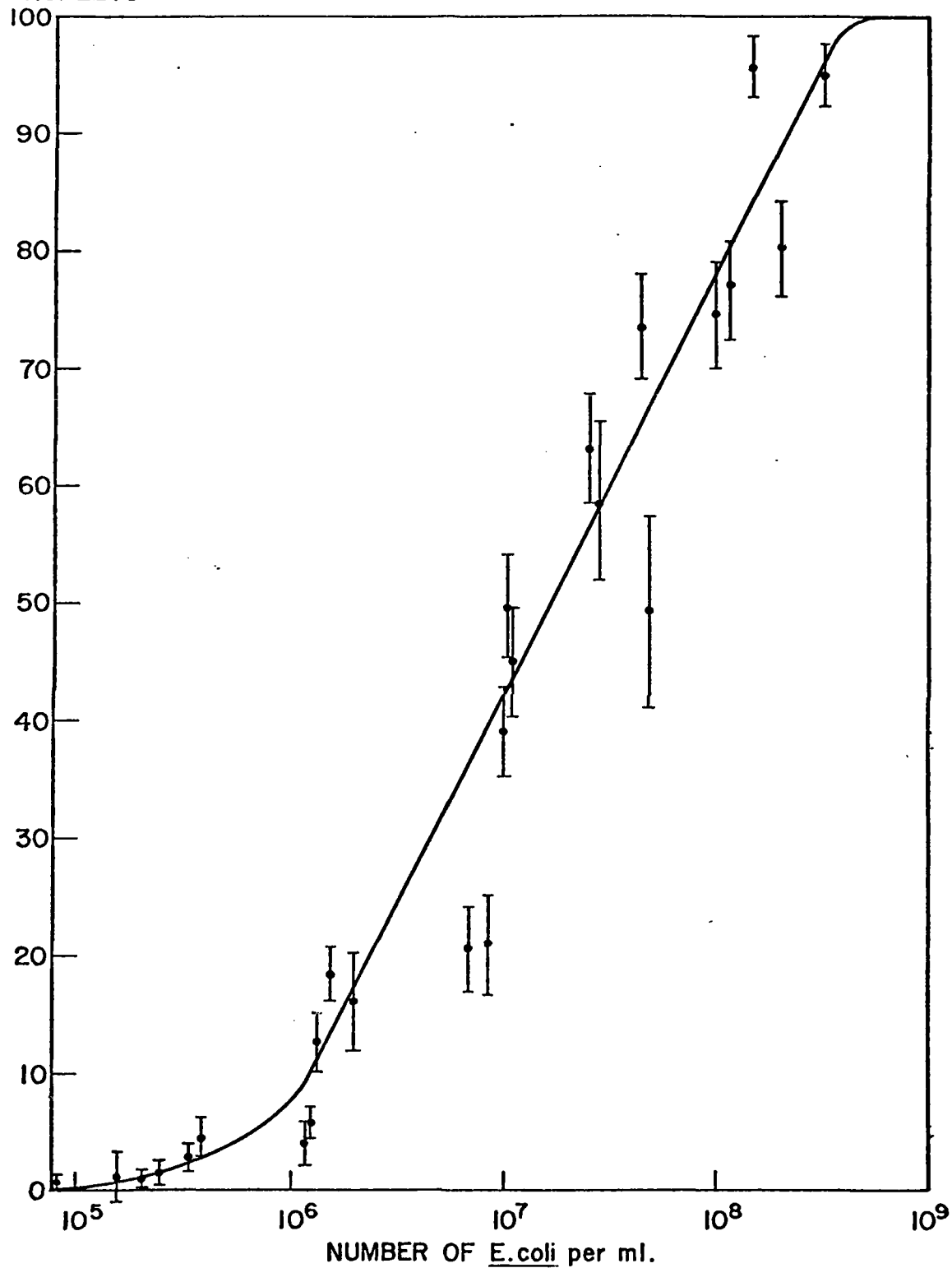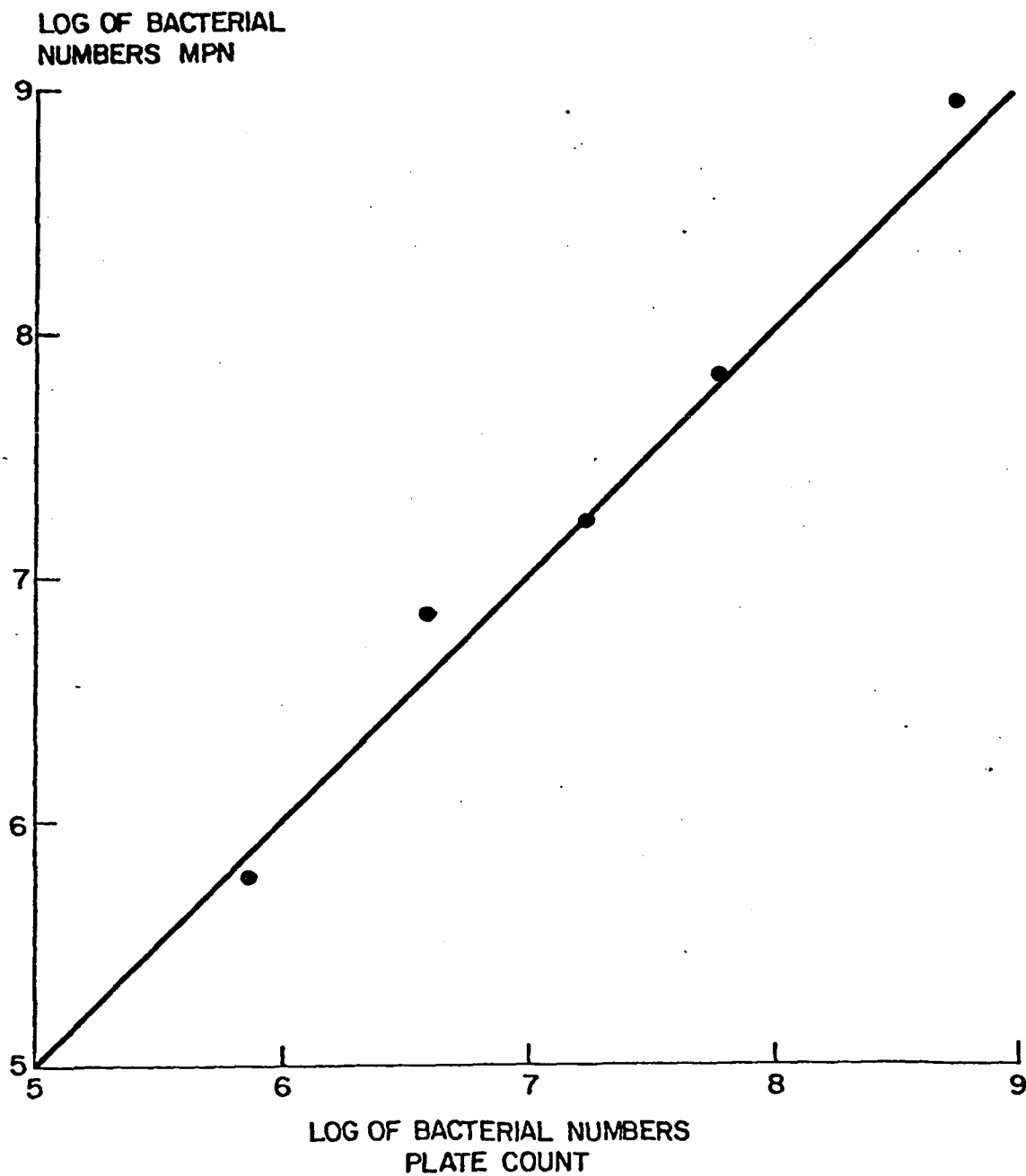
Figure 1

RELATIONSHIP BETWEEN THE PERCENTAGE OF FLUORESCENT
DROPLETS PER FIELD OF VIEW AND THE CELL DENSITY OF
E.coli DETERMINED BY PLATE COUNTS

FIGURE 2

LOG OF BACTERIAL
NUMBERS MPN

LOG OF BACTERIAL NUMBERS
PLATE COUNT

CORRELATION OF COLIFORM CELL DENSITIES DETERMINED
BY MPN AND PLATE COUNT METHODS

FIGURE 3

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Since it is a large step from determining the coliform numbers in cell suspensions of E. coli Neotype to the examination of field samples, the rapid coliform detection method was used to count coliforms in primary-treated sewage as well as E. coli suspensions.

A comparison of different coliform-counting techniques was made using a sewage sample and a cell suspension of E. coli Neotype as a source of coliform bacteria (See Table 1) The techniques employed were the plate counts on Eosin Methylene Blue and Mac Conkey's agar, the MPN determination using the multiple-fermentation tube technique and the membrane filter technique. The rapid detection method overestimated the number of coliforms in the sewage sample and underestimated the number in the E. coli suspension, compared to the other techniques. A possible reason for the higher coliform numbers with the rapid detection method is the presence of β-D-galactosidase positive organisms within the sewage that do not grow on the selective media used in the other techniques. In contrast with an E. coli suspension, repair mechanisms may operate with the plating and multiple-fermentation tube techniques that do not occur in the rapid coliform detection method. No conclusion, however, about the correlation between the five techniques can be established on the basis of a single determination. The results appear to lie within the error inherent in these techniques.

TABLE 1 – Comparison of different coliform-counting techniques to determine coliform numbers in a primary-treated sewage sample and an E. coli Neotype cell suspension.

Technique	Coliform Counts (organisms per ml)	
	Sewage Sample	E. coli Neotype
Plate Counts a) MacConkey's agar	3.2×10^6	1.5×10^8
b) Eosin methylene blue agar	3.4×10^6	1.3×10^8
MPN	2.5×10^5	1.8×10^8
Membrane filtration	6.0×10^4	1.3×10^8
Rapid Detection	8.2×10^6	6.5×10^7

The advantages of the rapid coliform detection method are 1) the specificity for bacteria with β-galactosidase activity at 35°C, i.e., coliform bacteria, 2) the rapidity of the method compared to current standard methods for coliform quantification and 3) the amenability of the method to automation. The disadvantages of the manual microdroplet spray method are 1) the high cell density (10^5 organisms per ml) required, 2) the necessity to concentrate low cell density coliform suspensions, and 3) the subjectivity of the fluorescent droplet counting.

A fundamental problem associated with rapid detection methods for coliform bacteria is the necessity to count very low cell densities. Either the coliforms are allowed to proliferate so there are sufficient numbers of cells for their detection, or the coliforms must be concentrated. The first approach increases the time taken to conduct the procedure, while the second approach is hampered by the lack of a presently acceptable concentration technique.

Two approaches were proposed to get around the problem of bacterial concentration. The first dealt with automating the microscope to scan a large area of a slide while counting fluorescent droplets via a photometer and counter. The second approach was to utilize a fluorescence flow cytometer and count every fluorescent particle. Because of the greater probability of achieving quantification at the low bacterial numbers level, emphasis has been placed on the flow cytometry approach. However, both techniques are described.

Semiautomation of the Fluorescent Droplet Counting Procedure

The principal difference between the manual and automated modes of detection and quantification are that the microscope stage is scanned in fixed increments by means of a mechanical scanning device rather than by simple manual manipulation. A photometer plus a recorder are used rather than visual readings for quantification. Thus, the Zeiss Standard 15 microscope with Farrand Spectrum Analyzer was equipped with a strip chart recorder to log the number and intensity of fluorescent microdroplets encountered while scanning prepared sample slides. Fluorescein-di-galactopyranoside (FDG) was added to E. coli suspensions which were induced for β-D-galactosidase activity and sprayed onto silicone coated slides. Each sample was horizontally scanned along its center diameter at a rate of 10-20 microns per second. The results demonstrated a number of distinct peaks of varying amplitudes representing the number of fluorescent microdroplets detected (See Figure 4).

FIGURE 4 – Photomultiplier Output of Microdroplets
Monochrometer Setting @ λ = 520 nm
Chart Speed = 10 cm/min.
Full Deflection = 100 mV
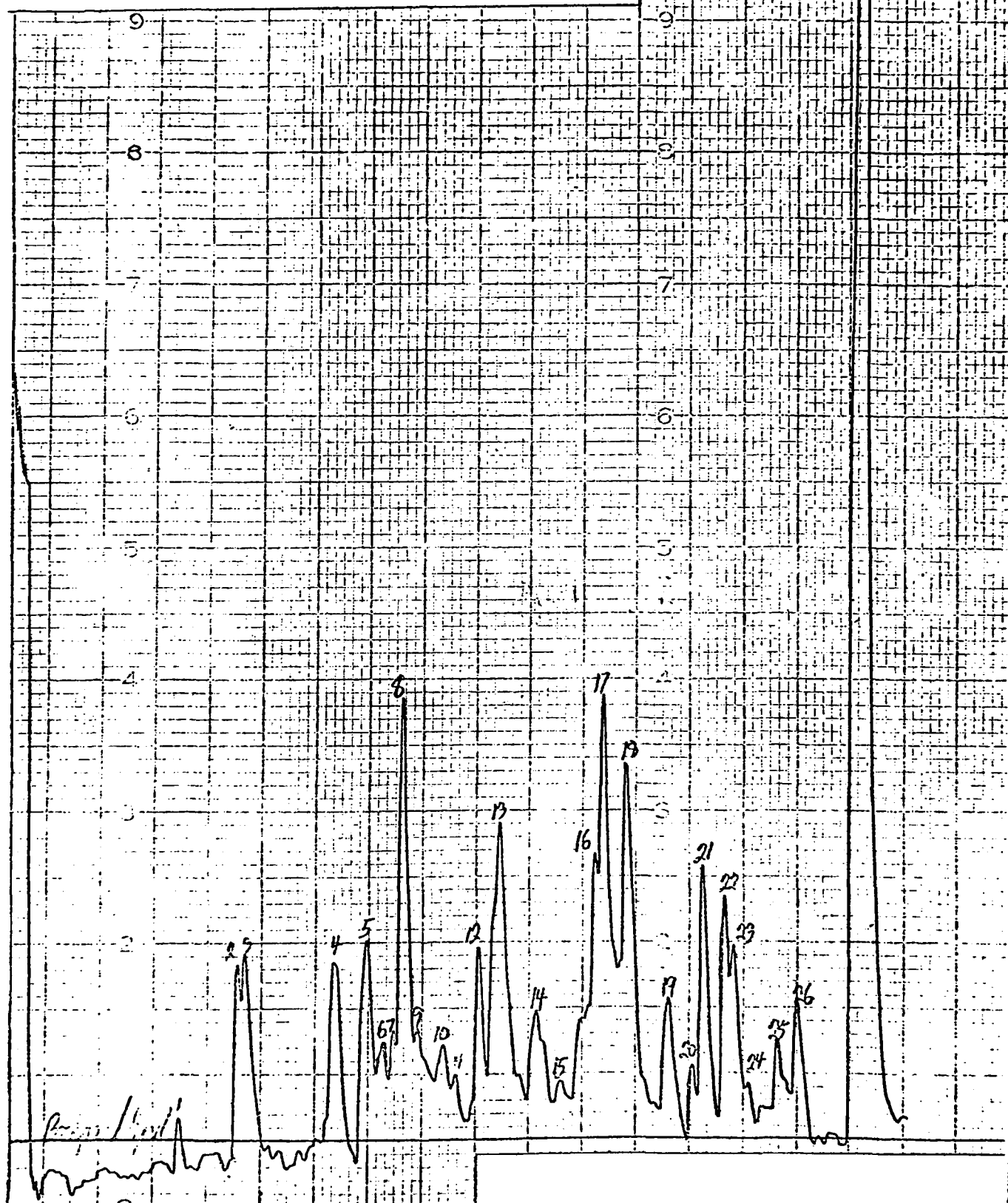
To obtain a correlation between numbers of fluorescent microdroplets and bacterial concentration, a number of parameters must be maintained, which include:
(1) Even aerosol dispersion
(2) Constant microdroplet density per field

The scanning of the field is accomplished completely in the fluorescent mode, therefore, microdroplets not containing fluorescein are not detected. As a result, the established standard procedure places a stringent limitation in parameter variability since a comparison between fluorescent and total numbers of microdroplets cannot be made.

Attempts at maintaining a homogeneous aerosol dispersion, as well as a constant microdroplet density, are difficult. It is currently infeasible to use the established procedure to obtain a correlation without the use of an automatic aerosol head designed for the parameters indicated. It should be noted that the semiautomated method is not the system of choice and that a number of recognized deficiencies exist, one of which is data correlation.

It was thought possible however, to improve the method utilizing a different approach, namely, the use of two fluorochromes. A second fluorochrome having the same excitation wavelength as fluorescein but exhibiting a sufficiently different emission wavelength could be employed. The technique involves the addition of a background fluorochrome to a bacterial suspension containing FDG. Upon aerosoling, all resultant microdroplets would contain the background fluorochrome while only those with coliforms would additionally contain fluorescein. As a result, the detection of both fluorescent and total microdroplet numbers could be accomplished. The slide would first be scanned at the emission wavelength of the background fluorochrome to obtain total microdroplet numbers and then at the emission wavelength of fluorescein to obtain fluorescent microdroplet numbers. The results could be expressed in terms of percent fluorescence which can then be correlated to bacterial concentration without critical dependence on the spraying technique.

The test protocol may be standardized a number of ways, two of which are:
(1) Scanning across the center of a well at a constant speed for a standard time duration.

(2) Alternately, scanning across the center wall until a total microdroplet count of 100 is obtained.

With this method many fields of view can be examined at one time with a high degree of reproducibility.

Preliminary investigations into the use of a two dye system were made. Acridine orange was the only fluorochrome initially on hand and was therefore tested as a possible background fluorochrome. A model system using pure reagents was used to simulate the Rapid Coliform Detection condition. A 200 μl aliquot of a 0.1% acridine orange solution was added to 200 μl of a 10^{-4}M solution of sodium fluorescein and then evenly sprayed onto a modified slide containing silicone. The slide was then sprayed a second time with a 0.1% solution of acridine orange. The final result was a slide which contained microdroplets of two types:

(1) Those containing only acridine orange (simulating microdroplets without coliforms).

(2) Those containing both acridine orange and fluorescein (simulating microdroplets with coliforms).

When observed under the fluorescent microscope one could readily distinguish between the two types of microdroplets. Those containing acridine orange fluoresced a bright orange while those containing acridine orange and fluorescein demonstrated a greenish-yellow fluorescence. It was noted, however, that when the slide was scanned with the Farrand Spectrum Analyzer, the emission band of acridine orange was too wide to be effectively discriminated from fluorescein with the monochromator.

We later tried other fluorochromes, including Evans blue, propidium iodide, and ethidium bromide. Only the latter proved to be useful. Preliminary suitability tests consisted of spraying a pure 1% ethidium bromide solution (E-B) onto silicone coated slides and observing under the fluorescent microscope. The resultant microdroplets displayed a bright orange-red fluorescence and negligible photo decay over a 15 minute period. In its pure state, E-B exhibited emission minima at 435 and 660 nm and an emission maximum at 576 nm. From this data it was evident that ethidium bromide could be differentiated from fluorescein but that the converse would prove to be difficult. Consequently, a series of more dilute E-B solutions were tested in the hopes of "narrowing the band width" at the expense of some loss in intensity. (See Figure 5). In subsequent trials it was found that the more dilute E-B solutions (0.1%, 0.01%, and 0.005%) did in fact effectively reduce signal overlap.

The next stage of our testing involved the induction of E. coli Neotype for β-D-galactosidase activity and adding E-B to the suspension as the background fluorochrome. A 200 μl aliquot of induced cells (A = 0.01≈10^8/ml) was added to 25 μl of fluorescein di-galactopyranoside (FDG) and 12.5 μl of a 0.1% E-B solution. The suspension was sprayed onto silicone coated slides and incubated for 15 minutes at 30°C. Observation under the fluorescent microscope demonstrated a distinction between microdroplets containing E. coli (positive) and those which did not (negative). Positive microdroplets fluoresced in a variety of color shades ranging from green to yellow-orange in response
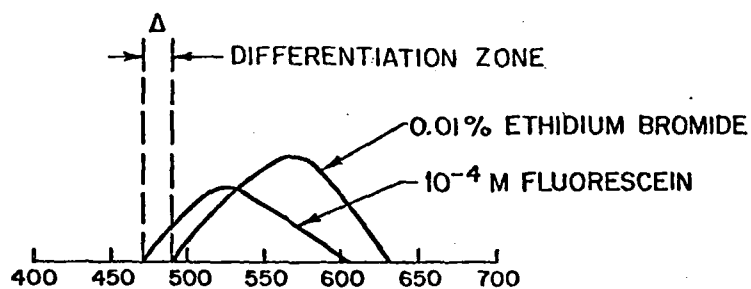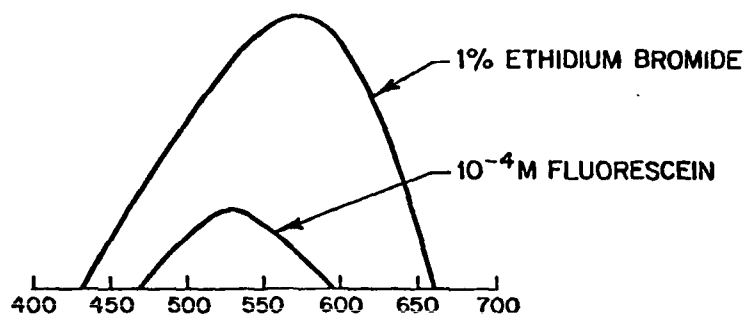
**EFFECTS OF CONCENTRATION ON
BANDWIDTH OF ETHIDIUM BROMIDE**

FIGURE 5

to the number of E. coli cells per microdroplet and the relative intensity of fluorescein with respect to E-B. Negative microdroplets fluoresced an orange-red. Therefore, a visual distinction could readily be made amongst the microdroplets, but was it possible to now find a wavelength which could block out the E-B emission and detect only fluorescein? To answer this question the output signal of the spectrum analyzer was connected to a strip chart recorder set at a chart speed of 10 cm/min. and 100 mV full deflection.

A prepared slide containing microdroplets of E. coli and having E-B as the background fluorochrome was scanned at a constant speed at a wavelength of 520 nm. The resultant peaks represented those microdroplets detected which contained both fluorescein and E-B, and therefore, the total number of microdroplets encountered along the scanning path. This first scan was continued for 2½ minutes and then terminated. At this point, the monochromator setting was changed to a wavelength of 485 nm to determine the number of microdroplets containing fluorescein. A second scan of the slide was conducted starting from the endpoint of the first and traversing back to the origin. The peaks obtained on the graph now represented the number of fluorescein-containing microdroplets only.

The endpoint of the first scan and the origin of the second scan were used as points of reference since they were equal to the same position on the slide. A standard distance was measured (25 cm) from each point respectively, and the total number of peaks per distance was recorded (See Figures 6 and 7). Since the two scanning distances overlap, they represent the identical scanning path traversed by both trials making their peak numbers comparable. The number of fluorescein-containing and total microdroplets encountered were counted from the graph. The results were expressed in terms of % fluorescence and compared to a previous graph obtained relating % fluorescence with cell density of E. coli.

The next trial using the semiautomated technique yielded 80% fluorescence for a cell suspension of approximately 10^8/ml based on the absorbance readings. This corresponded very well with 78% fluorescence extrapolated from the Manual Technique graph for a 10^8/ml E. coli concentration (See Figure 8).

The results indicate that the two dye system is potentially feasible and that a greater number of trials and refinement could improve detection. There is a limiting problem in the inability to detect microdroplets having a low fluorescein intensity. These appear orange even though a bacterium can be observed within the microdroplet.

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FIGURE 6

Total Microdroplet Detection

Ethidium Bromide Emission – Monochrometer Setting @ $\lambda = 520$ nm
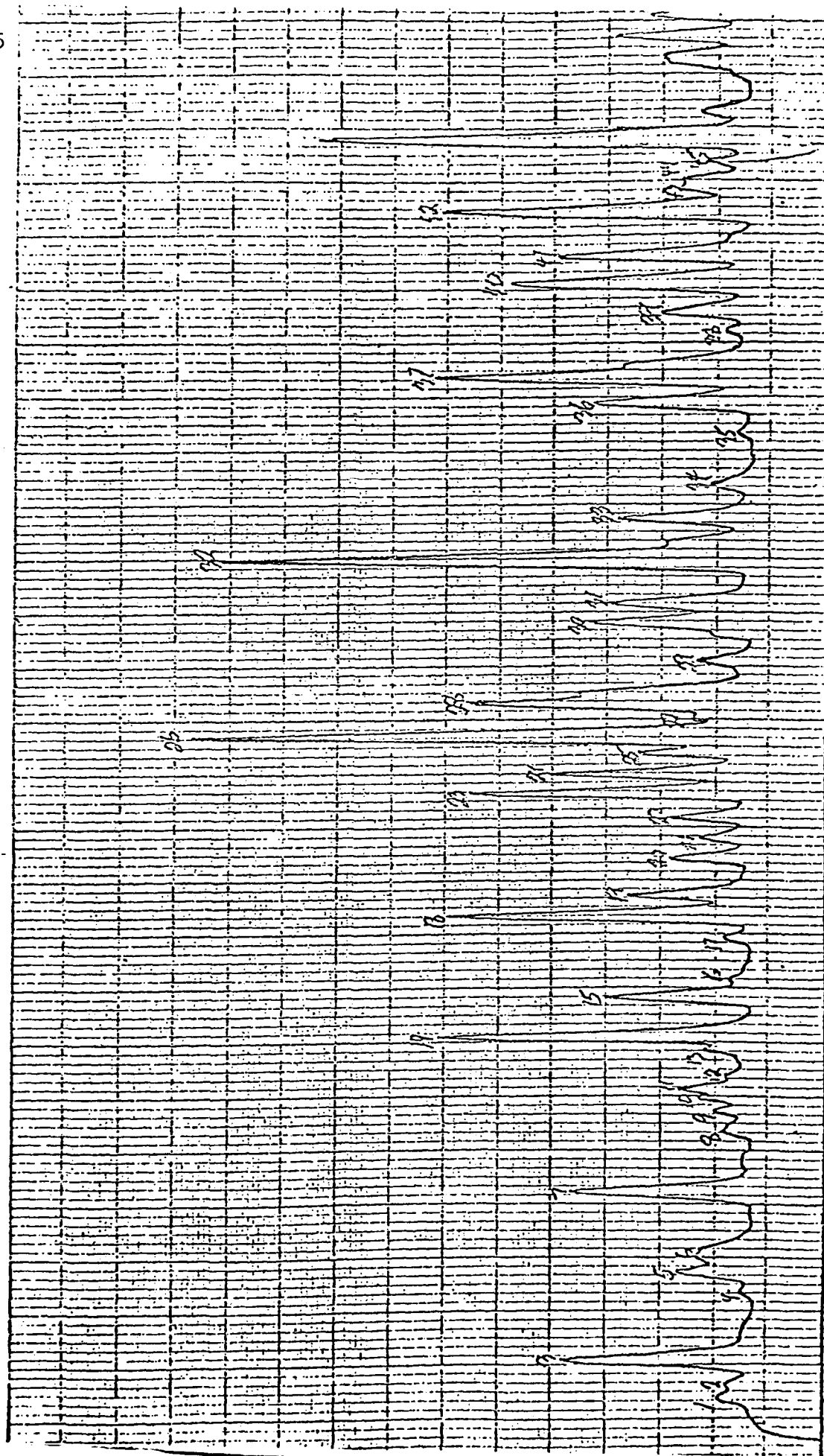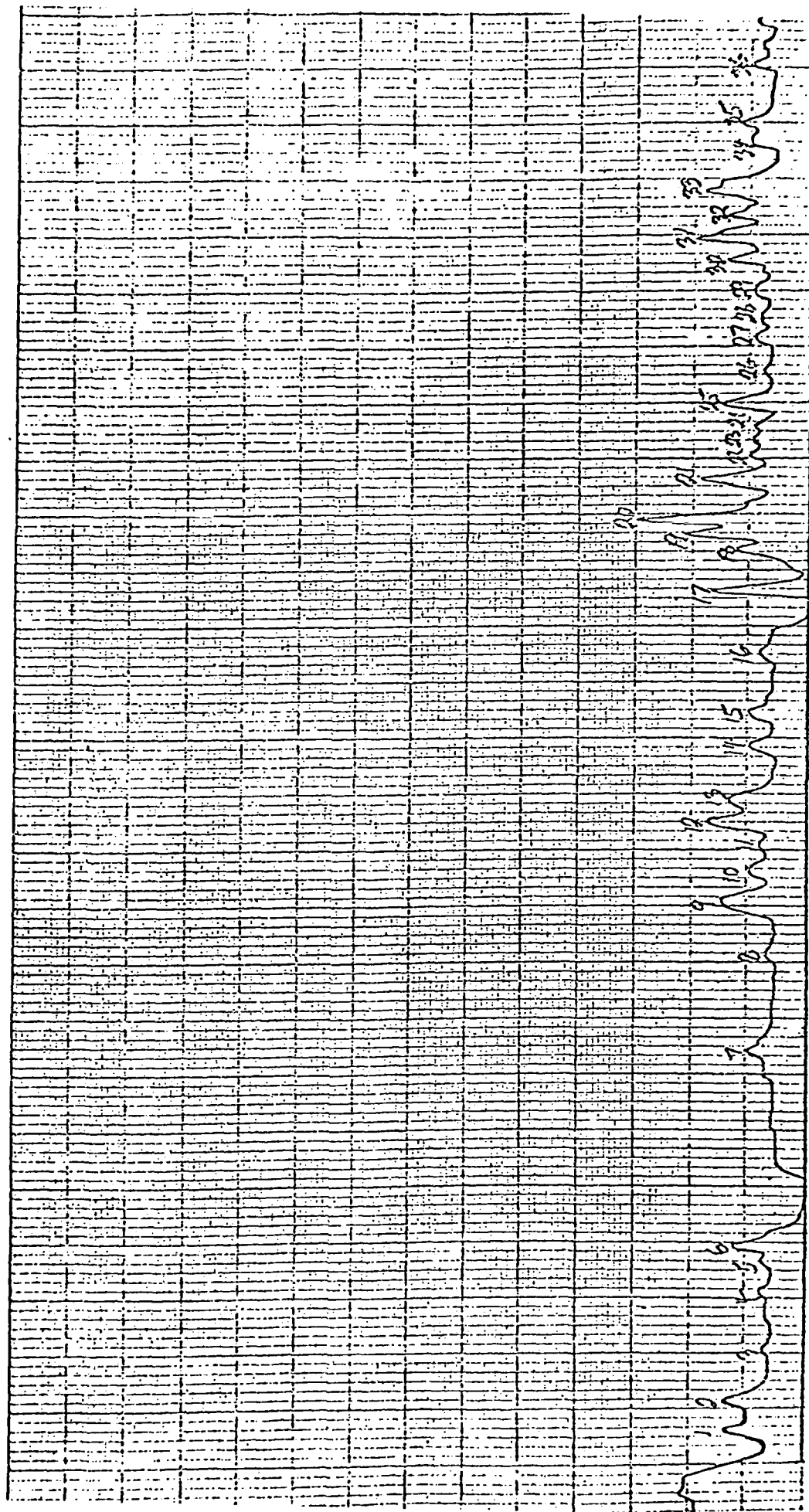
Chart Speed = 10 cm/min.   Full Deflection = 100 mV

FIGURE 7

Positive Microdroplet Detection
Fluorescein Emission Monochromator Setting @ λ = 485 nm
Chart Speed = 10 cm/min. Full Deflection = 100 mV
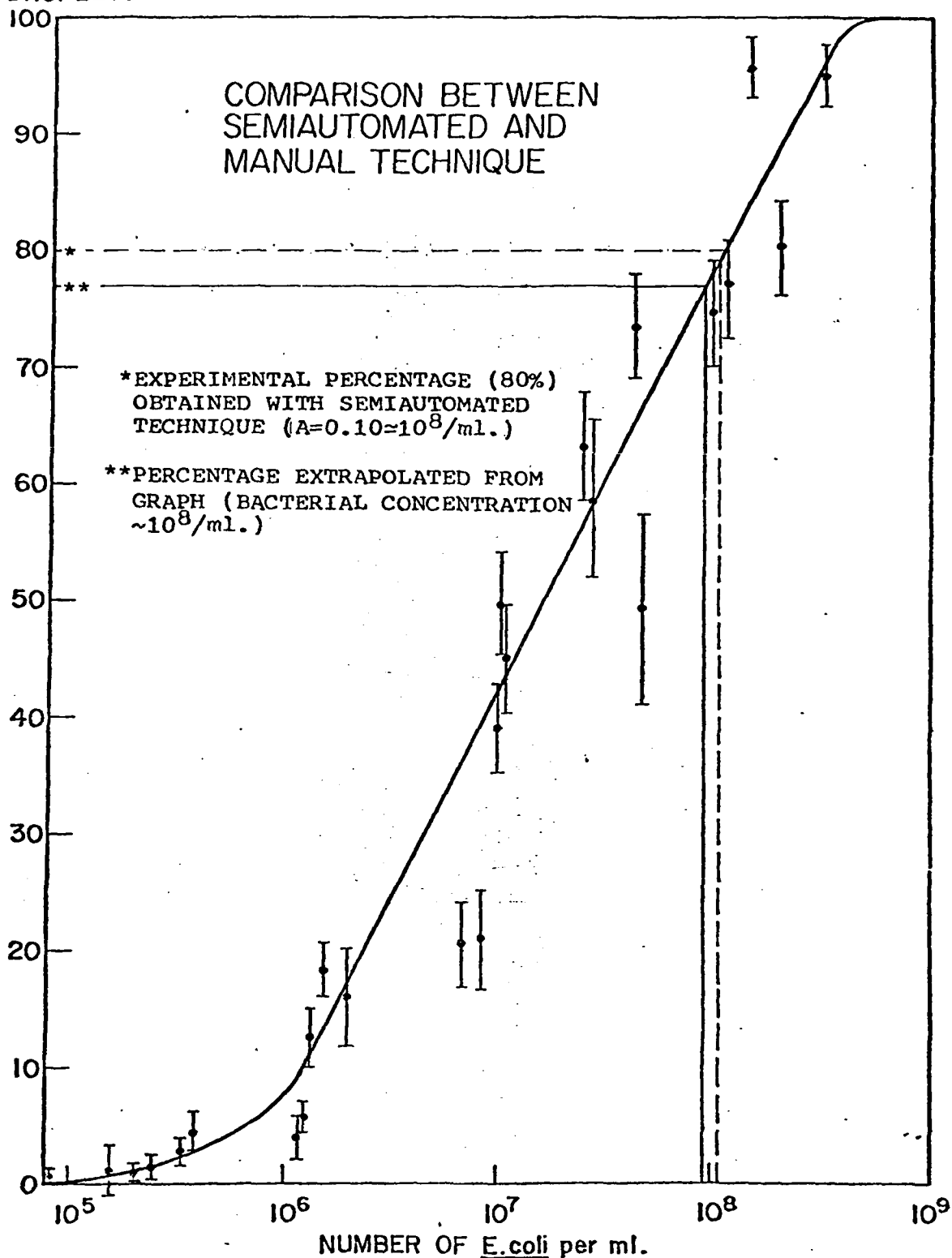
16

PERCENTAGE OF
FLUORESCENT
DROPLETS

COMPARISON BETWEEN
SEMIAUTOMATED AND
MANUAL TECHNIQUE

*EXPERIMENTAL PERCENTAGE (80%)
OBTAINED WITH SEMIAUTOMATED
TECHNIQUE (A=0.10≈10^8/ml.)

**PERCENTAGE EXTRAPOLATED FROM
GRAPH (BACTERIAL CONCENTRATION
~10^8/ml.)

NUMBER OF E.coli per ml.

RELATIONSHIP BETWEEN THE PERCENTAGE OF FLUORESCENT
DROPLETS PER FIELD OF VIEW AND THE CELL DENSITY OF
E.coli DETERMINED BY PLATE COUNTS

FIGURE 8

Validation of Fluorochromasia Detection Using A Fluorescence Flow Cytometer

After several delivery delays, we finally received our fluorescence flow cytometer in March of 1980 and project emphasis was switched to this technique. Several months were spent calibrating and optimizing the optical, flow and electronics subsystems for coliform detection and quantification.

Initial tests using relatively concentrated bacterial suspensions ($\sim 10^6$/ml) resulted in discouragingly low levels of detection ($\sim 1:10,000$). Consequently, a new approach was considered which involved the development of micelles within a bacterial suspension undergoing fluorochromasia. An article (Gratzel, 1980) described both significant increases in fluorescent intensity and photostability (increased fading time) when cyanine dyes were irradiated in micellar systems. Although fluorescein is not a cyanine dye, the principle of micellar systems may still apply. It is conceivable that micelles could be formed about individual bacteria undergoing fluorochromasia to the extent of retaining passively diffused fluorescein about the region of the cell surface. In effect, bacteria would act as nuclei for micelle formation resulting in an increase of fluorescent intensity per bacterium — the net result being a net increase in the level of detectability. Sodium lauryl sulfate was chosen as the candidate surfactant and used at its critical micelle concentration (the point of micelle formation from unassociated molecules of surfactant). The critical micelle concentration (CMC) was determined experimentally by taking conductivity measurements for a concentration series of sodium lauryl sulfate. Plotting equivalent conductivity vs normality$^{\frac{1}{2}}$, a break in the curve was obtained indicating the CMC. The extrapolated value was determined to be 10^{-2}N (See Figure 9).

The effect of a micelle system was evaluated by inducing a suspension of E. coli Neotype for β-D-galactosidase activity. Initial results, though by no means definitive, looked encouraging. Several months were spent investigating various reagents in an attempt to maximize fluorescence within a "bacterial micelle". Occasionally, results appeared to be promising, however, reproducibility of test results could not be obtained. The reproducibility problem, particularly at low concentration levels i.e., $\simeq 1,000$ bacteria/ml, masked any correlation of sample concentration and numbers of bacteria counted. There appeared to be an interference effect of unknown origin.

To approach the problem systematically, possible interference sources were divided into two main categories, i.e., instrumental and/or particulate. Noise signals may have been generated by the instrument itself at the point of the lamp source or photomultiplier tube. Alternately, there may have been particulates within the sample, characteristically fluorescent in the green region.
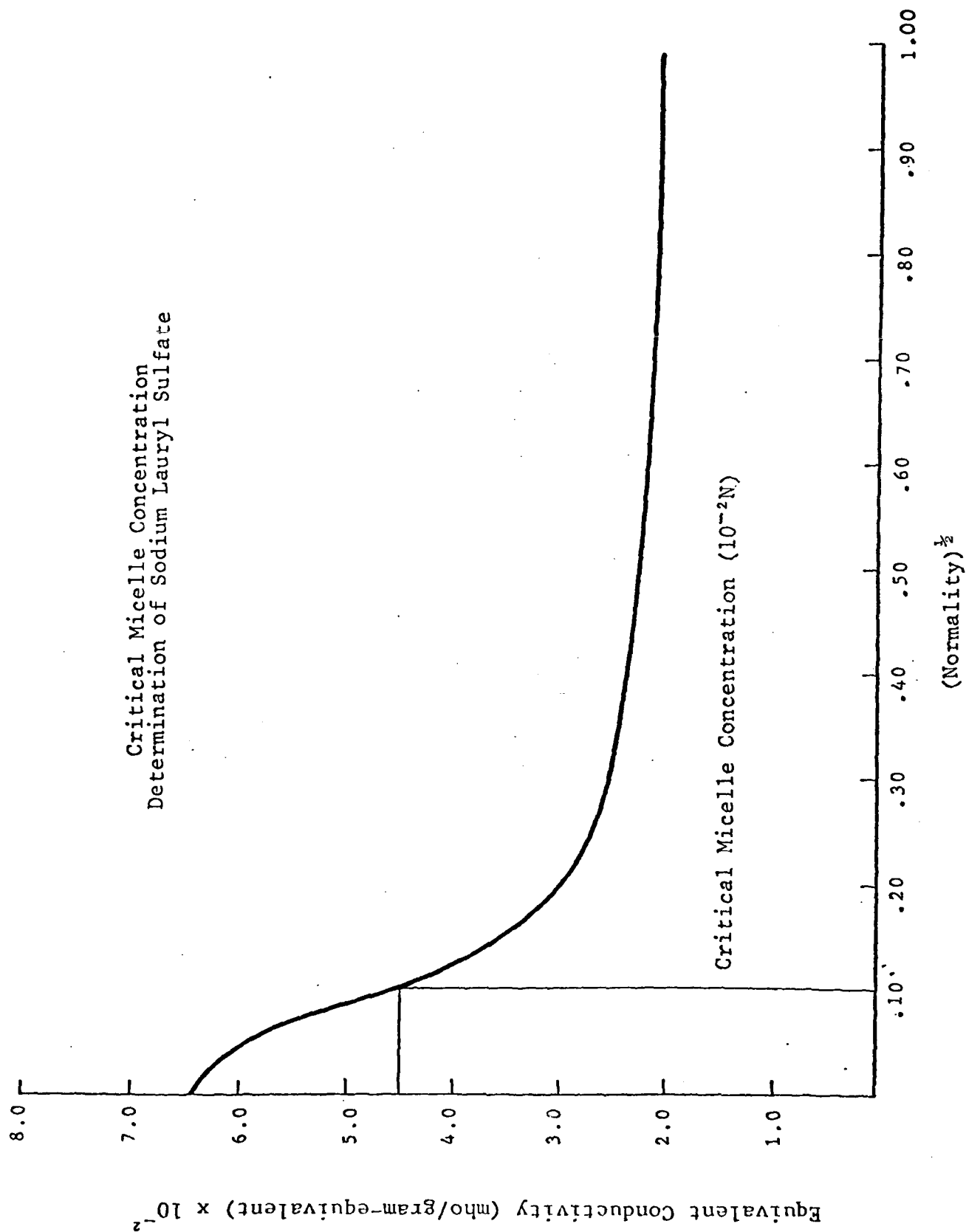
Critical Micelle Concentration
Determination of Sodium Lauryl Sulfate

Critical Micelle Concentration $(10^{-2}N)$

$(Normality)^{\frac{1}{2}}$

Figure 9

Equivalent Conductivity (mho/gram-equivalent) $\times 10^{-2}$

In all background noise studies conducted, average relative count rates/50 sec were used as a basis of comparison. This reflected the amount of time necessary to traverse an oscillo-scope's storage display offering a convenient method for analyz-ing and documenting data. Preliminary studies were directed towards the optical system of the instrument. The replacement of the mercury-arc lamp with two new mercury-arc sources demon-strated no improvement. The photomultiplier tube was found to be in working order through the use of standard beads at various concentrations. The instrument response showed a good correlation with concentration with a high degree of precision. The spectral characteristics of the filter elements were examined through the use of a Beckman spectrophotometer (Model 25) and found to be consistent with the manufacturer's specifications.

The role of possible fluorescent particulates present with-in the system was addressed. A variety of solutions was pre-pared (particularly lactate media) and passed through a 0.45μ Millipore filter. The results demonstrated a persistence of the background signal and in some cases a modest increase. A further precaution against particulates was the addition of 8μ filter tubes at the point of the reservoir inlet tubes. This additional safeguard, however, did not eliminate the background signals. The above observations indicated that particulates were not the cause and that other possibilities need be inves-tigated.

The possible presence of small air bubbles within the sam-ple, sheath, and transverse fluids, was then considered as the source of background signals. Since each stream flows through the optical path, a bubble passing through that area might re-sult in a signal. If this were indeed the source of signals, one would expect a highly aerated sample to have a significantly greater count rate than one which had been vacuumed.

To test this hypothesis carbonated water and vacuumed tap water were run through the instrument and the average of their count rates compared. The former was found to have a signifi-cantly greater count rate over the latter (5,680/50 sec vs 14/50 sec). Furthermore, when the carbonated sample was degassed for 10 minutes using a magnetic stir bar, the count rate decreased, as expected, to a level of 196/50 sec. It was also observed that an increase in background signals arose when the sample, sheath, transverse, and waste lines were tapped vigorously. The spurious patterns obtained were very similar to those wit-nessed with partially degassed carbonated water.

Additional studies further indicated that background sig-nals observed were the result of the release of dissolved air in the form of bubbles due to the vacuum system employed in the cytometer. To confirm this, the sample inlet tube was clamped off and the instrument turned on. Under these conditions only the transverse and sheath fluids, arising from the reservoir, passed through the detection zone. Any signal generated from

such an arrangement would indicate that the problem area, under these conditions, resided in the reservoir system and had nothing to do with sample particulates. Test results demonstrated that this was indeed the case. Another series of tests also demonstrated that air bubbles in the sample tube also produced bubbles that gave erroneous cell counts. We therefore set about modifying the flow system to eliminate bubbles.

The first flow system modification involved the use of hydrostatic pressure obtained by simply elevating the sample and reservoir supplies above the flow cytometer. Bubble generation was drastically reduced, but flow control was lost. We next modified the flow system by the use of low pressure shop air to pressurize the reservoir and sample bottle. This appeared to work satisfactorily.

With the completion of flow modifications, we again started testing the "bacterial micelle" approach. Test results were not encouraging. Our data at this point indicated that hydrolyzed fluorescein contained within bacteria, as a result of β-galactosidase activity, rapidly diffused into the external environment, unlike the cyanine dyes of Gratzel.

A paper entitled, "Efflux of β-Galactosidase Products from Escherichia coli " by Huber et al. (1980) dealt specifically with the fate of the enzyme hydrolysates of lactose. The following quote from their conclusions provides additional proof that hydrolyzed fluorescein undergoes rapid efflux, "The results of the study clearly show that when lactose is administered to E. coli cells, the vast majority of the products of β-galactosidase action on this sugar are found in the medium. This was the case with a variety of growth conditions and strains and it occurred regardless of the rate of product metabolism." The problems encountered with detection, as well as the observations indicated above led to the conclusion that individual bacteria would have to truly be encapsulated to achieve any significant degree of detection. This encapsulation can conceivably include fixing of the bacterial membrane as well as entrapment of a bacterium within a second interface.

Encapsulation of Bacteria Through the Use of Microemulsions

The use of emulsions of the w/o (water in oil) type was investigated as a means of encapsulating bacteria. Silicone oil was chosen as the candidate oil phase due to its proven nontoxic inertness and its characteristic nondiffusibility with respect to fluorescein. A preliminary evaluation of the water/ silicone oil emulsion technique was carried out using a dilute solution of fluorescein (10^{-5}M). A fluorescein emulsion was prepared by high speed, high shear mixing of a 1:9 water to oil mixture. The microdroplets formed were in the 10-100μ diameter range, and could readily be distinguished when observed under the fluorescence microscope.

Similar tests were made encapsulating coliform bacteria ($\sim 10^8$/ml) in an emulsion after they had been pretreated to hydrolyze FDG. Again, fluorescence was readily detected. Emulsified bacterial samples were introduced through the flow cytometer and for the first time fluorescent microdroplets were seen through the cytometer's flow system. These observations demonstrated that the use of emulsions to encapsulate bacteria was indeed feasible.

Further investigations centered on the development of relatively stable emulsions using minimal energy input and simple operating conditions. Initial efforts revealed that emulsions of increasing viscosities, when passed through the flow cytometer, produced pulsed signal interference of a regular nature. A silicone oil of lower viscosity (5 cp.) was used to minimize instrumental incompatibility. Additionally, the use of various surfactants and cosurfactants was investigated to increase emulsion stability.

The major surfactant and silicone manufacturers, including Rohm & Haas, BASF, Dow Corning, GAF, General Electric, and Union Carbide were contacted. Communications with their respective technical staff resulted in some suggestions as to surfactant selection, but little information regarding specific methodologies in the formation of emulsions of the water in oil type using silicone oils. The majority of industrial applications of silicone emulsions are of the oil in water type and unsuitable for our purposes. A systematic evaluation of potential surfactants, therefore, had to be conducted using a standardized procedure to screen a number of surfactants and cosurfactants chosen on the basis of chemical structure, HLB value (Hydrophile-Lipophile Balance) and suggestions from manufacturers.

Each candidate surfactant was added in a volume equal to the aqueous phase, to a test tube containing 4.5 ml silicone oil (GE's SF-96 @ 5 cps), 0.5 ml of a 10^{-5}M fluorescein solution, and 0.5 ml of cosurfactant (where used). In cases where a blend of two surfactants was used, the total volume of surfactant added was still equal to the volume of the aqueous phase (0.5 ml). Each mixture was vortexed for one minute and a 15μl aliquot transferred to a microscope slide for observation. The average emulsion diameter and distribution were estimated by examining several fields under low and high power (125X and 563X) using an eyepiece micrometer. Surfactants were also judged visibly in terms of degree of settling, flocculation, coalescence, and bulk phase separation. Surfactant combinations which demonstrated a significant coalescence and phase separation in less than ½ hours time were judged as inadequate.

The use of cosurfactants consisting of aliphatic alcohols was suggested in the literature for the enhanced stabilization of w/o emulsions. Therefore, parallel studies determining the effects of a variety of different alcohols on the action of a particular surfactant were also examined. A list of some of the surfactants and cosurfactants studied follows:

TABLE 2

Surfactants	Cosurfactants
Sodium Lauryl Sulfate (Sigma)	Propanol
Triton X-45 (Rohm & Haas)	Pentanol
Triton X-100 (Rohm & Haas)	Hexanol
Igepal CO850 (GAF)	Cyclohexanol
Tween 20 (Sigma)	Decanol
Tween 40 (Sigma)	
Tween 60 (Sigma)	
Tween 80 (Sigma)	
SF-1178-Experimental (General Electric)	
X2-3225C-Experimental (Dow Corning)	

Results from the study demonstrated that Dow Corning's experimental surfactant with an equal volume of hexanol as a cosurfactant was capable of forming a relatively stable emulsion which met our criteria. The use of a simple vortex for one minute resulted in an excellent, uniform distribution of microdroplets having diameters less than 10μ.

The next stage of the experimentation was focused on the elimination of the pulsing effect. One approach to the problem was to conduct a study to determine to what extent the surfactant (X2-3225C) concentration could be minimized and yet retain a fairly stable emulsion. Test results indicated that a 2% surfactant concentration was the lowest limit attainable without major compromises in average emulsion diameter or stability. Control studies with the flow cytometer using the lower surfactant concentration demonstrated a definite improvement in terms of detection, but some intermittent pulsing still prevailed.

The pulsing phenomenon was thought to be the result of a decrease in the sample flow rate, stemming from an increase in viscosity, causing a discontinuous sample stream. The hydrophobic nature of the emulsion with respect to the aqueous sheath stream may have also enhanced this effect. The net result was the formation of discrete globules of sample which passed through the focal plane of the flow chamber to produce pulsed interference signals. (See Figure 10).

It became apparent that the instrument's air pressurized fluid delivery system had limitations in maintaining constant flow rates and that other means need be investigated.
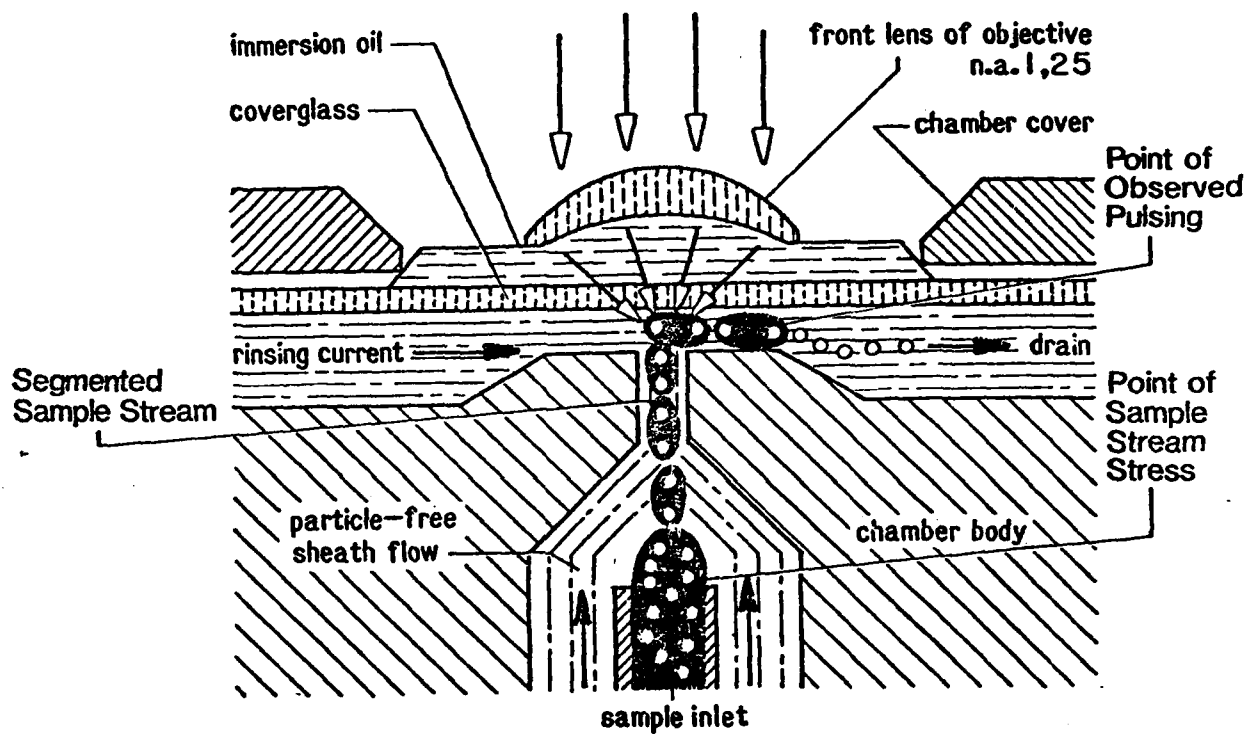
Sheath Flow Measuring Chamber.

FIGURE 10

The use of a polychannel peristaltic pump to independently control sample, sheath, and transverse flow rates entering the cytometer flow chamber was examined. Independent control of individual flow rates was accomplished by means of flow loops, outfitted with vernier handled metering valves, and connected by T-fittings to a reuse jar. Under this arrangement, maximum flow was maintained when the valve was fully closed (no diversion to reuse jar) and gradually decreased by opening the valve (increased diversion to reuse jar). Flow loops were applied only to the sheath and transverse lines.

Optimum flow rate settings were determined for each input stream using both deionized water and silicone oil to maintain a constant flow equilibrium. Vernier settings were recorded and utilized in subsequent sample trials. Fluorescein control emulsions ($\sim10^{-5}$M), simulating bacterial cells undergoing fluorochromasia, were run through the flow cytometer under the new arrangement. Results obtained from a number of trials demonstrated a complete elimination of the pulsing effect with no sheath flow reversal.

At this point, studies using E. coli Neotype samples optimally induced for β-D-galactosidase activity were resumed. Analysis of emulsions containing relatively high concentrations of bacteria ($\sim10^7$/ml) were found to elicit signals corresponding to only a fraction of the theoretical concentration. This was considered to be the result of multiple numbers of bacteria entrapped within individual microdroplets being counted as one. Subsequent studies conducted with lower bacterial concentrations ($\sim10^2$-10^3/ml) resulted in counts demonstrating a lack of correlation, with the greater majority being too high. A negative control emulsion of the substrate was run to determine the possibility of background signals arising from autolysis of the substrate. A 0.5 ml aliquot of FDG ($\sim5\times10^{-5}$M) was added to 4.5 ml silicone oil (SF-96-5), 100μl surfactant (X2-3225C), and 0.5 ml cosurfactant (hexanol). The mixture was vortexed for 1 minute and run through the flow cytometer. At a gain of 5.0, a count of 12,228/5 ml was obtained where there should have been none. It was evident that trace amounts of fluorescein arising from autohydrolysis of the substrate could be readily detected by the flow cytometer. This problem was not previously encountered due to a dilution step (1:100) incorporated in the original procedure to minimize the interference of the fluorescein. The present emulsion technique does not afford an opportunity at which a dilution of the sample can be performed. Another possible contributing factor is the relatively more intense emission provided by a 100W mercury-arc excitation source compared to the 100W halogen lamp used on our fluorescence microscope.

In addressing the substrate background noise problem a number of methods had been considered for its solution which included:
(1) Threshold and/or gain adjustment
(2) Further purification of substrate
(3) Increased permeability of bacterial membrane to substrate influx and fluorescein efflux.

Suppression of noise signals through threshold and gain adjustment proved unsuccessful. Studies conducted to increase membrane permeability using 20% isoamyl alcohol in one case and 10^{-2}N sodium lauryl sulfate in another did not demonstrate any significant improvement.

Purification of FDG was undertaken using several methods including H_2O extraction, recrystallization (both in ethanol and methanol), and KH_2PO_4 buffer extraction (pH = 7.0). The latter proved to be the most effective. Absorbance readings were taken @ 495 nm to determine the presence of fluorescein. A decrease in absorbance from 0.040 in the original preparation to 0.005 in the purified one demonstrated the effectiveness of the extraction. Furthermore, results obtained from negative control emulsions run through the flow cytometer, demonstrated virtually no background noise signals. A decrease in background level with increased purity can readily be observed in the three FDG preparations shown in Figure 11a, b and c.

A study using the newly purified substrate with two concentrations of E. coli Neotype (10^3/ml and 10^7/ml) induced for β-D-galactosidase activity was conducted. FDG was added to 2.0 ml of each of the two bacterial concentrations and thoroughly vortexed. A 0.5 ml aliquot of each concentration was separately transferred to duplicate test tubes containing 4.5 ml silicone oil, 300μl surfactant (increased from 2% to 6% for added emulsion stability), and 0.5 ml cosurfactant (hexanol). Each mixture was thoroughly vortexed and incubated @ 37°C for 30 minutes. During incubation, two negative control emulsions containing FDG at a final concentration $\simeq 4.6 \times 10^{-5}$M were run through the flow cytometer and found to elaborate virtually no counts (see Figure 12a and b). After incubation, sample emulsions were run through the flow cytometer for analysis. The results obtained revealed that the E. coli Neotype emulsions having final concentrations $\simeq 10^3$/ml were not detected with the flow cytometer after 30 minutes incubation @ 37°C. Sample emulsions having higher concentrations ($\sim 10^7$/ml), however, demonstrated detection rates of 0.3% and 0.2% for trials I and II, respectively. (See Figure 13a, b and c.)

SUBSTRATE CONTROL COMPARISONS
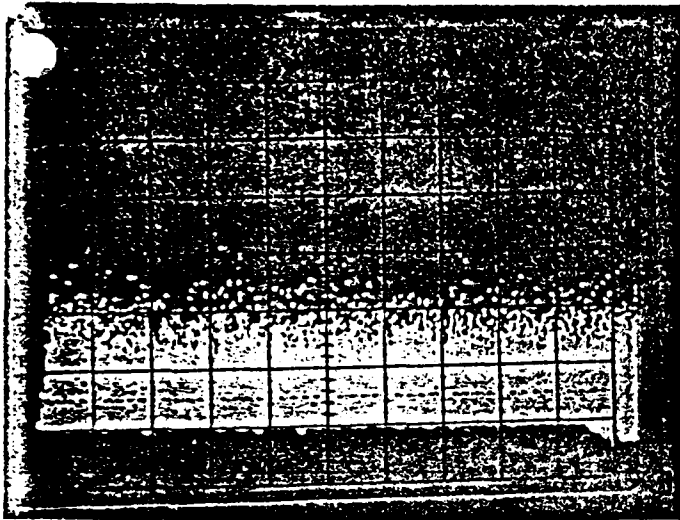
Original Substrate Preparation

Sample: Negative FDG Control Emulsion
$(\sim 5 \times 10^{-5} M)$

Gain: 5.0
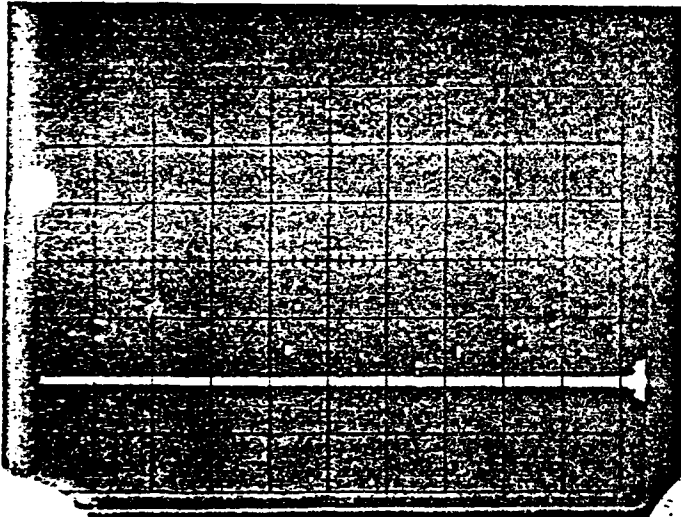
Sample Pump Speed: 2.0

Sheath Vernier: 0.0

Transverse Vernier: 4.0

Final Count: 12,228/5ml

Voltage: 10mV/cm

Time Base: 500 ms/cm

Figure 11a

New Substrate Preparation (unpurified

Sample: Negative FDG Control Emulsion
$(\sim 5 \times 10^{-5} M)$

Gain: 5.0

Sample Pump Speed: 2.0
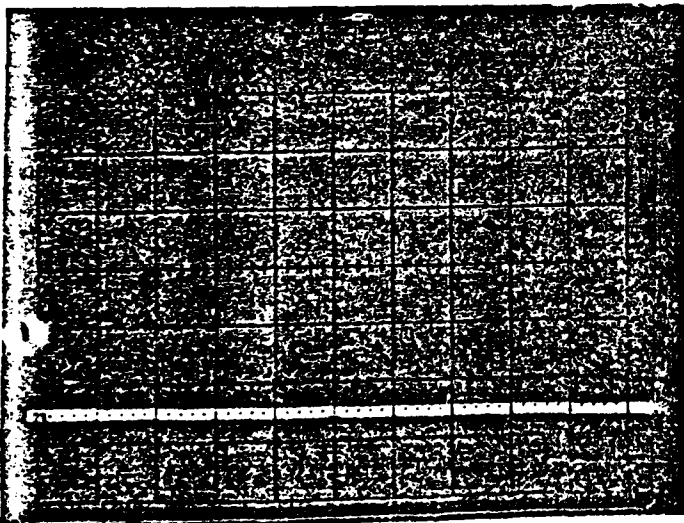
Sheath Vernier: 0.0

Transverse Vernier: 1.0

Final Count: 50/5ml

Voltage: 10mV/cm

Time Base: 500 ms/cm

Figure 11b

KH_2PO_4 Buffer Extracted Substrate Preparation

Sample: Negative FDG Control Emulsion
$(\sim 4.6 \times 10^{-5} M)$

Gain: 5.0

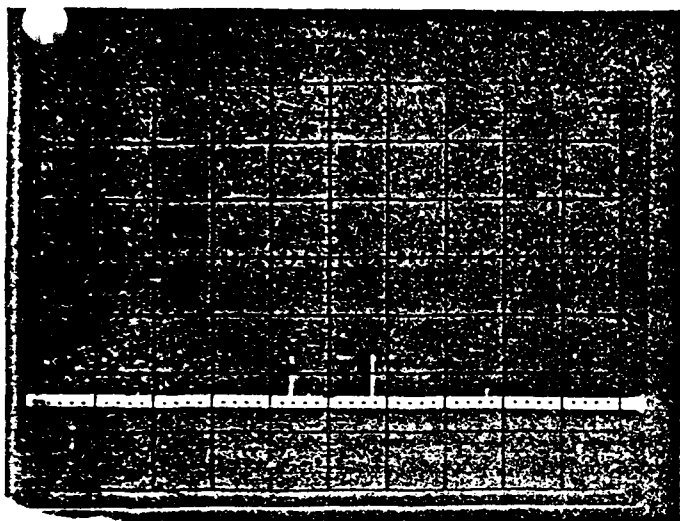Sample Pump Speed: 2.0

Sheath Vernier: 0.0

Transverse Vernier: 0.0

Final Count: 5/5ml

Voltage: 10mV/cm

Time Base: 500 ms/cm

Figure 11c

MICROEMULSION STUDY

Negative Control-Trial I

Sample: Purified FDG Emulsion
$(\sim 4.6 \times 10^{-5} M)$

Gain: 5.0

Sample Pump Speed: 2.0
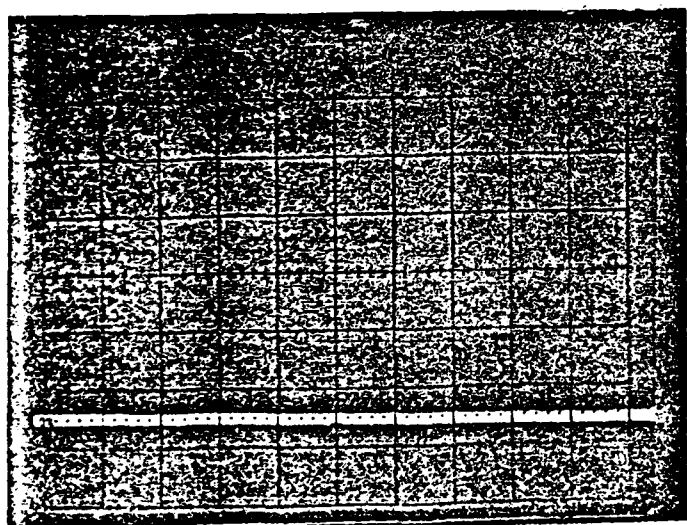
Sheath Vernier: 0.0

Transverse Vernier: 0.0

Final Count: 15/5ml

Voltage: 10 mV/cm

Time Base: 500 ms/cm

Figure 12a

Negative Control-Trial II

Sample: Purified FDG Emulsion
$(\sim 4.6 \times 10^{-5} M)$

Gain: 5.0

Sample Pump Speed: 2.0

Sheath Vernier: 0.0
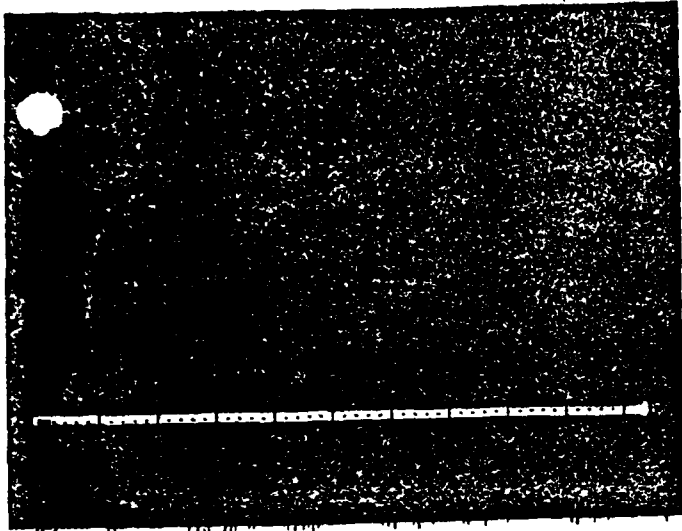
Transverse Vernier: 0.0

Final Count: 5/5ml

Voltage: 10 mV/cm

Time Base: 500 ms/cm

Figure 12b

Low Bacterial Concentration ($\sim 10^3$/ml)

Sample: E. coli Neotype Emulsion
Gain: 5.0
Sample Pump Speed: 2.0
Sheath Vernier: 0.0
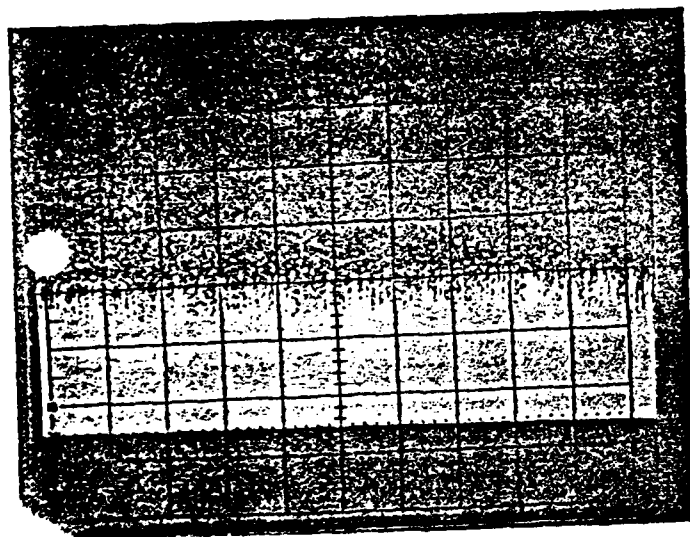Transverse Vernier: 0.0
Final Count: 3/5ml
Voltage: 10 mV/cm
Time Base: 500 ms/cm

Figure   13a

High Bacterial Concentration ($\sim 10^7$/ml)
Trial I

Sample: E. coli Neotype Emulsion
Gain: 5.0
Sample Pump Speed: 2.0
Sheath Vernier: 0.0
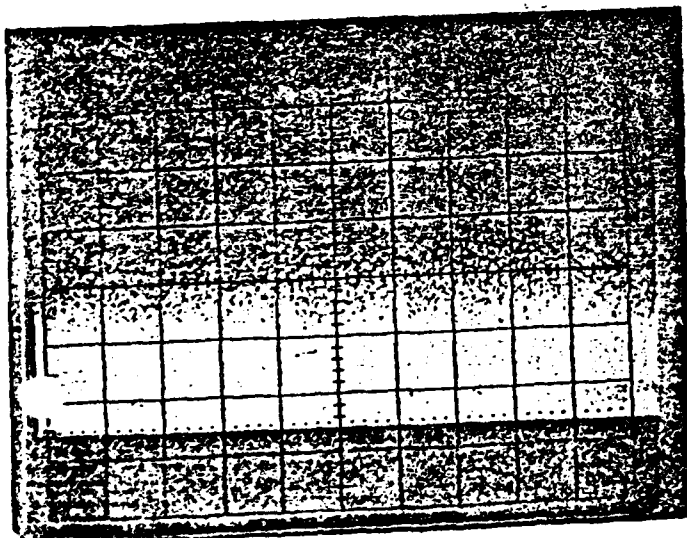Transverse Vernier: 0.0
Final Count: 143,761/5ml
Voltage: 10 mV/cm
Time Base: 500 ms/cm

Figure   13b

High Bacterial Concentration ($\sim 10^7$/ml)
Trial II

Sample: E. coli Neotype Emulsion
         (Analyzed ~ 15 Minutes after
          Trial I)
Sample Pump Speed: 2.0
Sheath Vernier: 0.0
Transverse Vernier: 0.0
Final Count: 105,736/5ml
Voltage: 10 m V/cm
Time Base: 500 ms/cm

Figure   13c

The problem of lack of detection was traced to 2 factors:
(1) the ratio of flow in the sample stream to that of the sheath
and transverse streams was far from optimal and (2) the gain
and threshold i.e., signal to noise level controls, on the in-
strument were not optimized. These factors were evaluated by
utilizing microdroplets of various concentrations of fluorescein
simulating coliform bacteria. Detection and quantification were
both enhanced orders of magnitude when sample and sheath flow
streams were brought into the correct ratio range and gain set-
tings increased appropriately.

SUMMARY

Work was continued on the validation of the rapid coliform detection method using a manual technique with both laboratory and field samples. The technique was reproduced from previous contractual efforts for familiarization purposes, due to a change in project personnel. Correlations were obtained with E. coli Neotype suspensions ranging in concentration from 10^5-10^8/ml. Additional studies, conducted with field samples (cesspool), demonstrated a correlation coefficient of $r^2=0.97$, using the manual technique.

A semiautomated approach to quantifying microdroplets sprayed onto silicone coated slides using a Zeiss Standard 15 fluorescence microscope equipped with a Farrand spectrum analyzer, a Zeiss scanning stage, and a motorized joy-stick control, was re-examined. The use of a 2-dye system, with 0.01% ethidium bromide as the background fluorochrome for the detection of negative microdroplets (without bacteria), proved successful in quantifying microdroplets while remaining in the fluorescent scanning mode. Although this procedure reduced operator fatigue and provided a somewhat more rapid and standardized approach to quantifying fluorescent microdroplets, it did not afford the ability of detecting lower concentrations of E. coli Neotype ($<10^5$/ml).

The use of an Ortho model ICP-22 fluorescent flow cytometer to detect fluorochromasia elaborated by E. coli Neotype was explored. Data indicated that fluorochromasia, per se, was below the resolving capabilities of this instrument even after extensive instrumental and procedural modifications. It became apparent that the rate of fluorescein efflux from the bacterial cell was too rapid to permit effective levels of accumulation to occur. As a result, the direction of the research effort was redirected onto methods of containing fluorescein within the region of individual bacteria. Preliminary studies conducted with membrane modifiers to contain fluorescein within the confines of the bacterial cell envelope were unsuccessful. Investigations into the use of emulsions however, were promising, resulting in the pursuit of that avenue.

Emulsions of the w/o (water in oil type) were investigated using silicone oil as the continuous phase. Initial studies involved the high speed homogenization of bacterial suspensions with silicone oil (1:9). Although emulsions could be obtained in this way, they were relatively unstable, with relatively wide ranging microdroplet diameters. An extensive screening of surfactants and cosurfactants to increase emulsion stability and reduce microdroplet diameter to <20µ was undertaken. The final outcome of this effort was the development of a methodology for forming relatively stable w/o emulsions using a low viscosity silicone oil under simple vortexing conditions.

The introduction of emulsions into the flow cytometer created flow stream difficulties, characterized by pulsing of the sample signal. The independent delivery of all fluid streams leading to the instrument's flow chamber by means of a poly-channel peristaltic pump was found to correct this situation. Subsequent studies, conducted with E. coli Neotype induced for β-D-galactosidase activity, demonstrated that preparations of high concentrations of bacteria could be detected but that at lower concentrations correlations with total signal counts were poor. During this period of testing, analysis of negative controls, consisting of a 1:10 dilution of the substrate as the aqueous phase, revealed that the flow cytometer was detecting trace amounts of fluorescein arising from a new problem, auto-lysis of the substrate. Purification of the substrate (FDG) by means of H_2O extraction, recrystallization, and KH_2PO_4 extraction was performed to eliminate background noise. A relatively pure substrate with virtually no background noise was ultimately obtained. Subsequent studies conducted with E. coli Neotype emulsions resulted in detection rates of 0.2 and 0.3% for bacterial concentrations $\simeq 10^7$/ml. Analysis of lower bacterial concentrations ($<10^3$/ml), however, were inconclusive.

It was determined in a following series of tests that the cause of the poor detectability and quantification were due to nonoptimized sample and sheath flow rates and low gain settings.

CONCLUSIONS AND RECOMMENDATIONS

Although the program described herein covered a period of 15 months, approximately 12 months were spent awaiting delivery and correcting unsuspected deficiencies in the flow cytometer. Thus, only 3 months of actual technique development took place. During this 3 month development period, additional, but not insurmountable problems in detection level sensitivity were noted. However, on the positive side, coliforms were definitely detected by the flow cytometric method. Optimization of various parameters to improve detection level remains to be completed.

The fluorescence enzyme detection technique holds promise of revolutionizing the field of microbiology, much as it did for clinical chemistry. Bacterial identification by enzymes specific to various classes of bacteria, as demonstrated by the presence of galactosidase in coliforms, appears to be a valid approach. Buttressed by automatic counting and identification by flow cytometry, it should be feasible to rapidly identify and quantify a wide variety of bacterial marker organisms (e.g. coliforms) plus bacterial pyrogens and pathogens. Therefore, it is strongly recommended that development of the technique be continued and expanded.

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